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Assessment of GPR30, a Seven Transmembrane-spanning Estrogen Receptor, as an Oncogene

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14. ABSTRACT

Our prior work has linked the seven transmembrane estrogen receptor GPR30 to specific estrogen binding (Thomas et al, 2005), rapid estrogen action (Filardo and Thomas, 2005) and the development of metastatic breast cancer in man (Filardo et al, 2006). To further address the role of GPR30 in experimental breast cancer biology, transgenic mice were created for the purpose of overexpressing wild type or active GPR30 in the mammary gland using the mouse mammary tumor virus tissue specific promoter. Two suitable candidate founder mice with stably integrated wild-type GPR30 were generated (T6-1A and T6-2E). We were unsuccessful in our attempts to generate an active GPR30 allele as assessed by in vitro assays outline in this proposal during this time frame. A no cost extension was requested to further propagate the wild-type mice and to analyze their capacity to develop mammary adenocarcinoma.

15. SUBJECT TERMS
membrane receptor, estrogen, transgenic mice

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Introduction.

Breast tumor growth and survival is strongly influenced by estrogen and decisions regarding appropriate adjuvant therapy for patients with breast cancer are largely determined by the measurement of known estrogen receptors (ERs) in primary tumor biopsy specimens. However, it has long been suspected that receptors other than the known estrogen receptors may promote estrogen action. Recent findings by our lab (1-4), and others (5-8), has shown that the seven transmembrane receptor (7TMR), GPR30, promotes specific estrogen binding and biochemical signaling and in addition is linked to tumor progression in man. To further address the role of GPR30 in experimental breast tumor biology, we have proposed to generate transgenic mice capable of overexpressing wild type or active GPR30 using mammary gland specific promoters.

Body.

Task 1. To evaluate the impact of hyperexpressed wild-type and CAM GPR30 on mammary duct branching and predisposition for the development of invasive breast cancer.

Selection of MMTV promoter over WAP promoter for conditional expression of HA-GPR30.

Conditional expression of transgenes in the mammary gland has been accomplished by numerous investigators using transcriptional regulatory elements derived from either the whey acidic protein (WAP) or the mouse mammary tumor virus (MMTV) (7). Since we had prior experience expressing a transgene under the transcriptional control of the whey acidic protein (WAP) promoter, here we proposed to similarly create a WAP-regulated hemagluttinin (HA)-tagged GPR30 transgene. During the interim between the submission of this proposal and its acceptance, we became concerned with a few minor potential concerns with the WAP expression vector that did not necessarily preclude its use, but made us reconsider, and select the MMTV promoter. Namely, while very strong expression was observed with the WAP transgene, not all mammary duct epithelia expressed the WAP transgene, and variation was observed in the percentage of ducts that were positive in progeny bred from the same founder. *We did not see this as significant deviation from the original statement of work to switch to the MMTV promoter and therefore proceeded.*

For this purpose, a MMTV expression vector that has been used for the construction of transgenic mice was employed. HA-GPR30 gene was assembled by PCR stitching and inserted into both MMTV (MMTV-SV40-Bssk; ref 9) and CMV expression vectors (see *task 2*, below). Expression of a functional HA-GPR30 protein was confirmed in HEK-293 cells (**figure 1).** The integrity of the MMTV-HA-GPR30 transgene was confirmed by restriction endonuclease mapping and DNA was purified by density centrifugation on a cesium chloride gradient.

Generation of HA-GPR30wt mice.

Purified MMTV-HA-GPR30 plasmid DNA was then linearized with restriction endonuclease *Sal I* and microinjected into eggs and transgene insertion was verified by PCR analysis of DNA extracted from tailsnips

taken from 24 mice. From this analysis, two female mice (T6-1A and T6-2E) were identified that contained the transgene (**figure 2**). These mice were then bred against CD-1 male mice and F1 progeny mice were yielded that had a transgene frequency that approached the predicted allelic distribution of 1/4 (+/- x -/-). Select transgene positive F1 mice were bred against CD-1 mice and similarly roughly 1/4 of the F2 offspring were also shown to inherit stably integrated HA-GPR30 transgene.

Generation of HA-GPR30CAM mice.

Four different mutants were generated with the goal of generating an active form of GPR30. By alanine scanning mutatgenesis, single substitutions were made in three contiguous domains of basic charge in the intracellular domain proximal to transmembrane VI (amino acid residues 222-232). Mutants were expressed in HEK-293 cells and basal adenylyl cyclase activity was assessed. No significant difference was observed in basal adenylyl cyclase activity between each of the mutants and wild type GPR30 (**figure 3**).

Task 2. Wild-type and CAM GPR30 will be tested for their capacity to promote cell biological changes in breast cancer cells.

CMV-HA-GPR30 was transfected into GPR30-deficient human MDA-MB-231 breast cancer cells in order to assess it biological influence on breast cancer cells *in vitro*. HA-GPR30 transfected MDA-MB-231 cells showed enhanced capacity to form anchorage independent colonies relative too vector transfected controls in response to estrogen stimulation (**figure 4**). Moreover, MDA-MB-231 (HA-GPR30) cells showed enhanced migration (haptotaxis) in Boyden chambers in response to estrogen stimulation **(figure 5)**. No significant difference was observed with regards to their capacity to proliferate in response to estrogen stimulation (**figure 6**).

Key Research Elements.

Two lines of mice (T6-1A and T6-2E) expressing stably integrated HA-GPR30 transgenes were generated.

Reportable Outcomes.

None.

Conclusions.

Two lines of mice (T6-1A and T6-2E) harboring stably integrated HA-GPR30 transgenes were generated. We were unsuccessful in our attempts to generate an active GPR30 allele as assessed by in vitro assays. A no cost extension was requested to further propagate the HA-GPR30 wild-type mice and to analyze their capacity to develop mammary adenocarcinoma. These animals represent potential interesting models for studying the role of this previously unappreciated estrogen receptor in breast cancer biology.

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Appendices.

None.

Supporting data.

Figures 1-6.

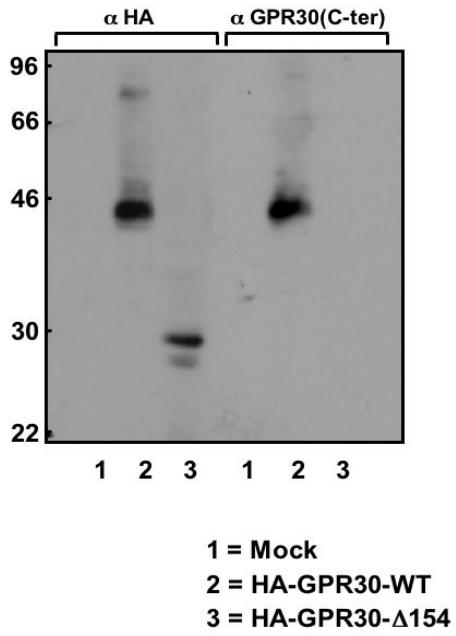

Figure 1. Expression of HA-GPR30 protein. Total protein (25 μg) from HEK-293 cells transfected with: pcDNA-3.1neo(+) vector, HA-GPR30, or C-terminally truncated HA-GPR30 immunoblotted with anti-hemagluttinin (HA) or GPR30 C-TER peptide antibodies. Molecular mass standards are indicated at left (kDa).

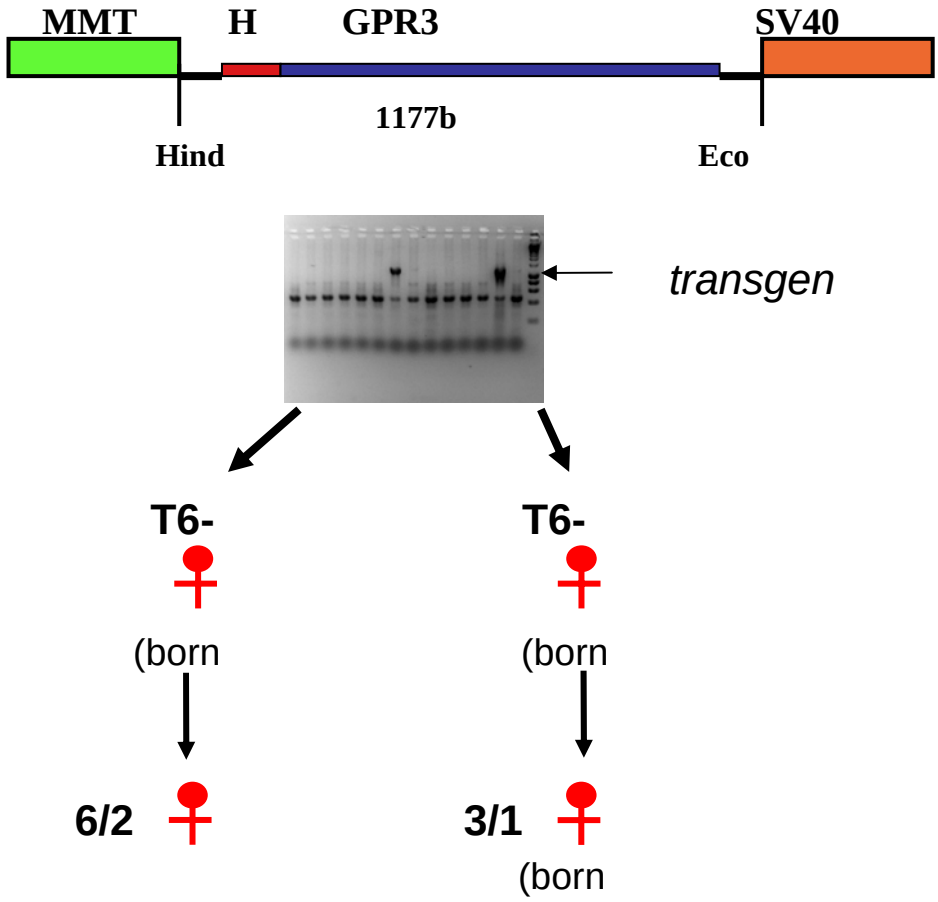

Figure 2. PCR analysis of tail DNA confirming HA-GPR30 wt founder and progeny. Founder mice T6-1A and T6-2E harboring the HA-GPR30 transgene expressed from the MMTV-BssK-SV40 expression vector from Phillip Leder's lab were identified by PCR analysis of tail DNA. T6-1A and T6-2E were bred with CD-1 males to evaluate the heritablity of the HA-GPR30 transgene. In the F1 generation, 6 of 20 offspring from T6-1A founder and 3 of 15 offspring of the T6-2E founder were confirmed as transgene positive females.

Structure of the intracellular domain proximal to transmembrane segment VI of human GPR30

TM5 TM6

GLCYSLIVRVLVRAHRHRGLR PRRQKALRMIL AVVLVFFV

Elimination of basic charge (target BBBB and BBXXB)

XXRVLVRAHRHRGLRP RRQKALRXXX Wild-type
XXBXXXBXBBBBXXBX BBXBXXBXXX Wild-type

XXBXXXBXBAHBXXBX BBXBXXBXXX R222A
XXBXXXBXBBABXXBX BBXBXXBXXX H223A
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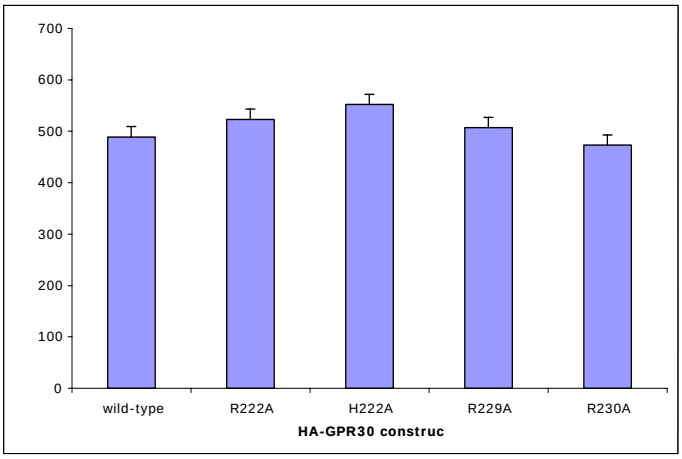

Figure 3. Strategy to generate a constituitively active mutant of GPR30. By alanine scanning mutatgenesis, single substitutions were made in three contiguous domains of basic charge in the intracellular domain proximal to transmembrane VI. Mutants were expressed in HEK-293 cells and basal adenylyl cyclase activity was assessed. No significant difference

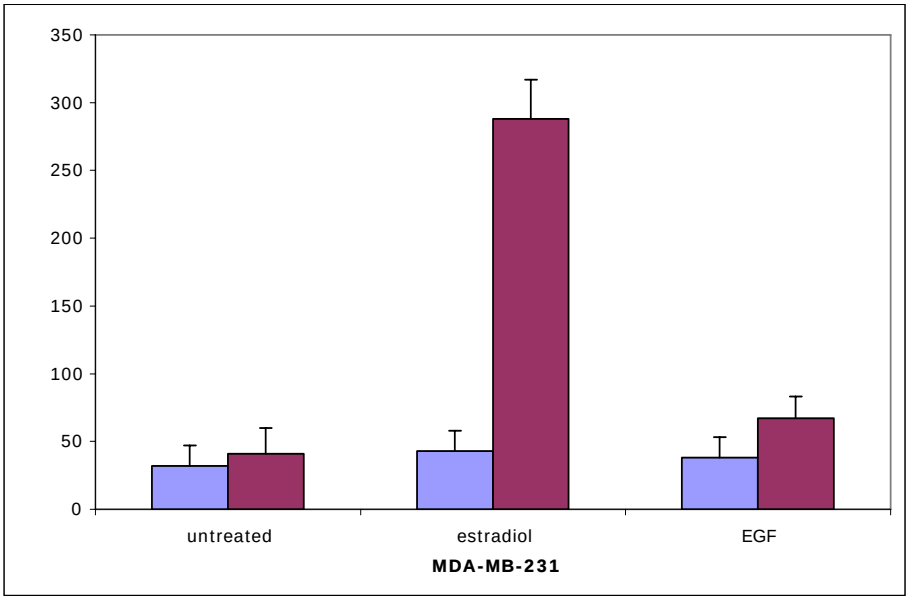

Figure 4. GPR30 stimulation promotes anchorage-independent growth. GPR30 deficient MDA-MB-231 human breast cancer cells were stably transfected with vector or HA-GPR30 and were tested for their capacity to form colonies in semi solid medium (0.35% agarose) supplemented with 17β-estradiol (10 nM) or EGF (1 ng/ml).

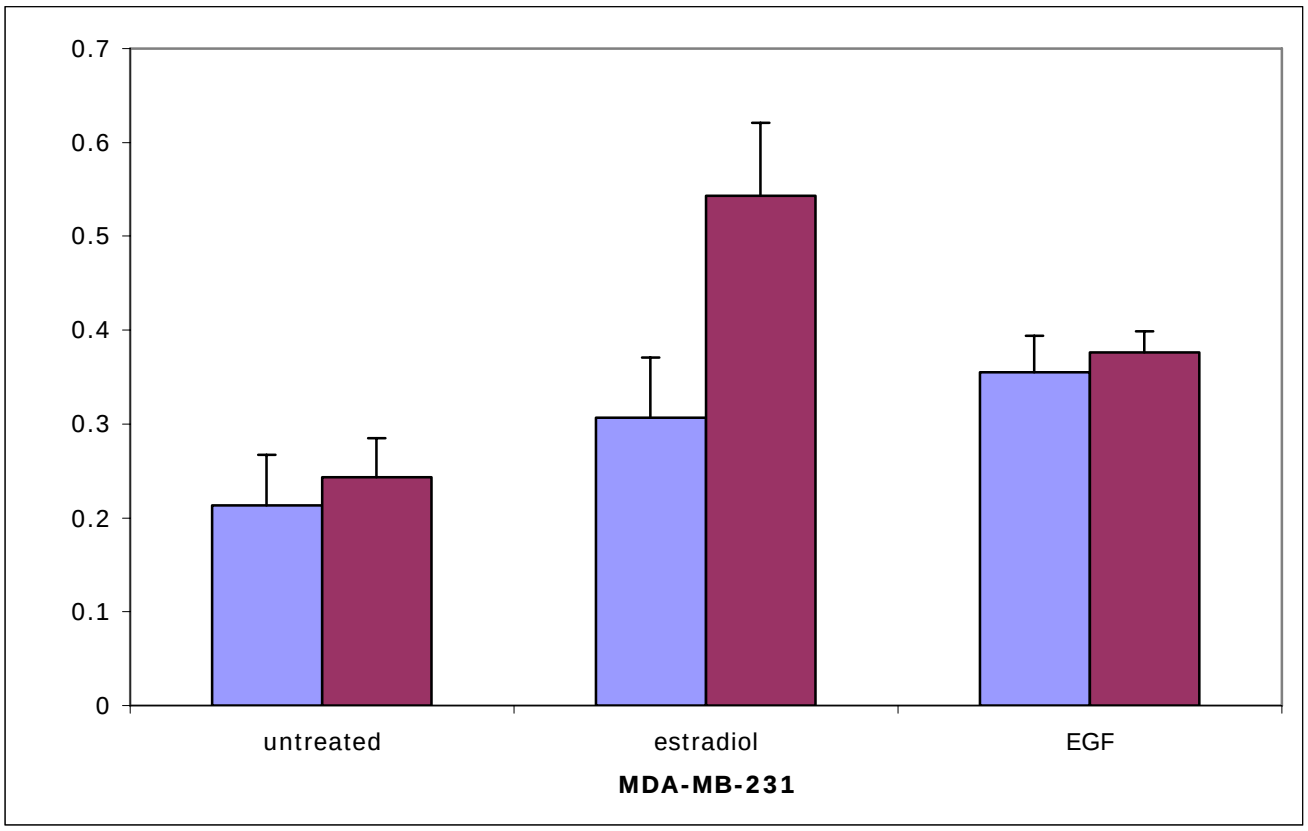

Figure 5. GPR30 stimulation promotes haptotaxis on fibronectin-coated substrata. GPR30 deficient MDA-MB-231 human breast cancer cells were stably transfected with vector or HA-GPR30 and tested for their capacity to migrate on fibronectin coated substrata using a Boyden chamber assay. One hundred thousand cells were seeded into the upper reservoir and stimulated with 17β-estradiol (10 nM), EGF (1 ng/ml), or vehicle. Following a 4 hour interval, cells remaining in the upper reservoir were removed and cells that migrated to the lower surface of the polycarbonate membrane were stained with crystol violet. The dye was eluted and quantified spectrophotometrically.

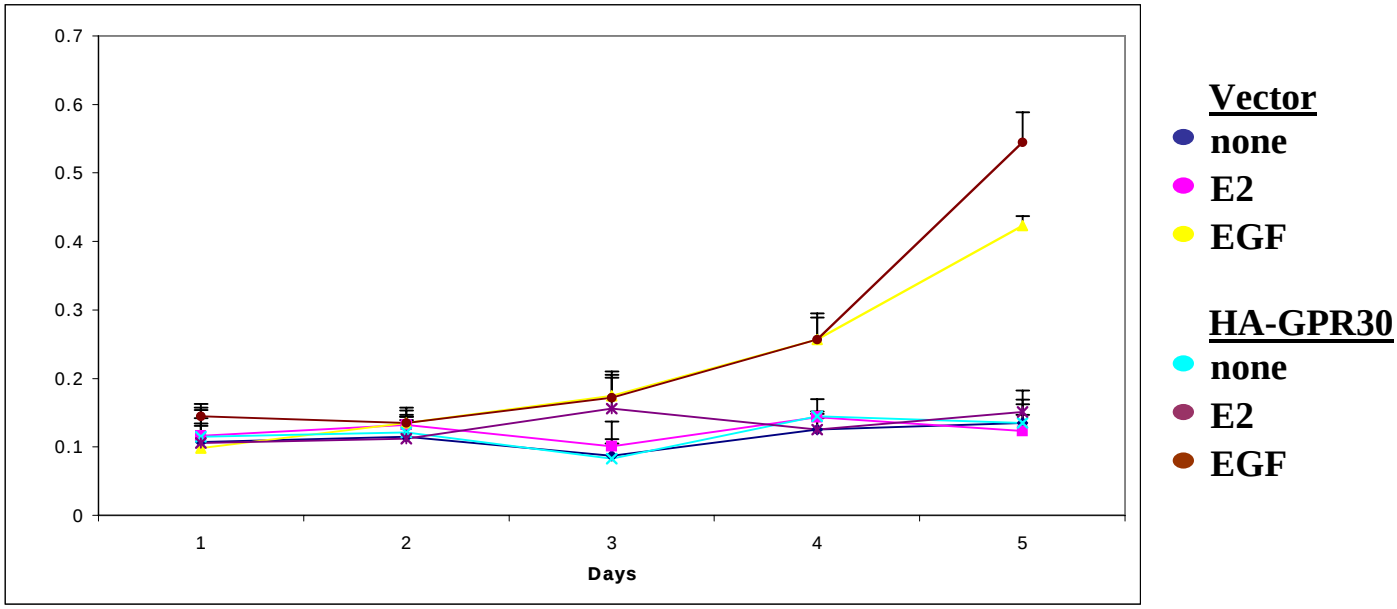

Figure 6. Growth kinetics of MDA-MB-231 cells expressing HA-GPR30. Serum starved MDA-MB-231 cells stably transfected with vector or HA-GPR30 were grown in 0.01% charcoal stripped fetal bovine serum containing no additives or supplemented with EGF (10 ng/ml) or 17β-estradiol (10 nM). Cell number was determined by MTT.